BRAIN COMMUNICATIONS

Bortezomib at therapeutic doses poorly passes the blood–brain barrier and does not impair cognition

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The 26S proteasome inhibitor bortezomib is currently used to treat multiple myeloma but also is effective in the treatment of antibody-mediated autoimmune disorders. One clinical concern is bortezomib's toxicity towards the (central) nervous system. We used standardized neuropsychological testing to assess cognitive function in six patients with myasthenia gravis and systemic lupus erythematodes before and after treatment with a mean cumulative dose of 9.4 mg m^{-2} bortezomib. In addition, cognitive performance was measured in adult C57Bl/6 mice after treatment with a human equivalent cumulative dose of 15.6 mg m^{-2}. Bortezomib concentrations were analysed in the human CSF as well as the brain tissue and serum of adult C57Bl/6 mice at various time points after the injection of 1.3 mg m^{-2} bortezomib with liquid chromatography–tandem mass spectrometry. Neither patients nor mice showed signs of cognitive impairment after bortezomib therapy. Bortezomib concentrations in the human CSF and murine brain tissue reached only 5–7% of serum concentrations with comparable concentrations measured in the hippocampus and the neocortex. Five-fold higher concentrations were needed to damage neuronal cells *in vitro*. In conclusion, penetration of the intact blood–brain barrier by bortezomib is low. Overall, our data show that bortezomib is a safe medication in terms of central nervous system toxicity.

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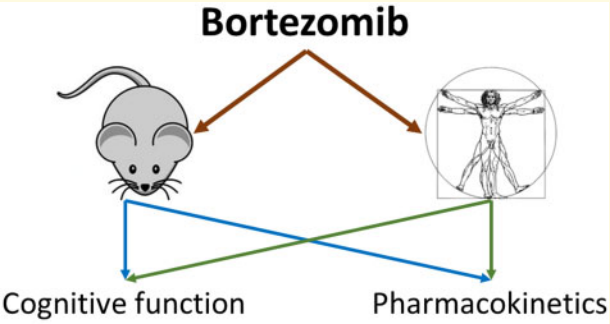
Abbreviations: BTZ = bortezomib; NSC = neural stem cells

Graphical Abstract



Introduction

Bortezomib (BTZ) is a 26S proteasome inhibitor currently used to treat multiple myeloma. BTZ induces the apoptosis of antibody-producing short- and long-lived plasma cells while sparing B cells (Alexander *et al.*, 2018). The former are often responsible for therapy-refractory stages in autoimmune conditions. Clinical data from patients with N-methyl-D-aspartate (NMDA) receptor antibody encephalitis (Scheibe *et al.*, 2017), systemic lupus erythematodes (Zhang *et al.*, 2017), primary Sjögren syndrome (Jakez-Ocampo *et al.*, 2015) or thrombotic thrombocytopenic purpura (Yates *et al.*, 2014) suggest that treatment with BTZ can lead to the rapid decrease in antibody titres, disease regression and better clinical outcome. However, one major concern is BTZ's toxicity towards the nervous system. BTZ-induced neuropathy is well described and occurs in 36–64% of patients with myeloma (Boehmerle *et al.*, 2015). CNS side effects are less well characterized, but diffuse symptoms such as cognitive dysfunction, fatigue and neurasthenia have been described following BTZ treatment in patients with myeloma (San Miguel *et al.*, 2006). The aim of this study was to measure BTZ concentrations in murine and human CNS and investigate BTZ's influence on cognition.

Materials and methods

A detailed description of this section is presented in the Supplementary material.

Clinical trial

Four patients with myasthenia gravis (three females and one male) and two patients with systemic lupus erythematodes (both female), each receiving a cumulative dose of 5.2–10.4 mg m^{-2} BTZ according to TAVAB trial protocol (NCT02102594) (Kohler *et al.*, 2019), were assessed for cognitive function prior to BTZ and 1–2 weeks after the last injection.

Assessment of cognition

We used a battery of five standardized and validated tests to assess different cognitive domains: verbal cognition was measured with the verbal learning and memory test (Thiel *et al.*, 2016). We used the Rey–Osterrieth complex figure test to examine visuo-spatial learning and memory (Shin *et al.*, 2006). Working memory was assessed with the digit span test (GrÉGoire and Van Der Linden, 1997). For concentration, processing speed and mental flexibility, the trail making test was used (Tombaugh, 2004). Executive function was measured with the Stroop test (MacLeod and MacDonald, 2000). Patients were asked to state as many items from one category within 1 min to assess semantic fluidity.

Pharmacokinetic analysis

CSF was collected twice via a lumbar draining system from a BTZ-treated patient with Caspr2 receptor antibody encephalitis at 30, 60, 90, 120, 240, 360, 720 and 1440 min after subcutaneous injection with 1.3 mg m^{-2} BTZ and snap frozen in liquid nitrogen. Two mice each were sacrificed at 5, 10, 15, 30, 60, 120, 240, 720 and 1440 min after the injection of 0.4 mg kg^{-1} BTZ (human equivalent dose of 1.3 mg m^{-2}) intraperitoneally. Blood samples were centrifuged, and serum was snap frozen in liquid nitrogen. Samples were prepared for analysis with liquid chromatography–tandem mass spectrometry very similarly to a previously described protocol (Clemens *et al.*, 2014).

Cell culture

Adult murine neural stem cells (NSC) of 2- to 4-week-old C57Bl/6 mice were prepared and maintained as described previously (Kronenberg *et al.*, 2010). Cell viability and caspase activity were measured with commercial assays as described previously (Boehmerle and Endres, 2011).

Behavioural analysis in mice

Mice received three injections of 0.4 mg kg^{-1} BTZ intraperitoneally per week for 4 weeks (human equivalent cumulative dose of 15.6 mg m^{-2}). Afterwards, the Morris water maze, Y-maze and novel object recognition test were conducted as described previously (Huehnchen *et al.*, 2017; Miedel *et al.*, 2017).

Statistical analysis

The conduction of the animal studies and preparation of the manuscript followed ARRIVE guidelines. Statistical analysis was performed using Prism v8.0 (GraphPad Software, San Diego, CA, USA). Gaussian distribution was checked using Shapiro–Wilk normality test. Normally distributed data were analysed with Student's *t*-test or one-way ANOVA with Sidak *post hoc* analysis and are shown as mean ± SEM. Mann–Whitney *U*-test, paired Wilcoxon test and Kruskal–Wallis test with Dunn's method were used for data that failed normality test and are presented as median with interquartile ranges. $P < 0.05$ was considered statistically significant.

Data availability

The dataset generated and analysed in the present study is available on Mendeley Data (Huehnchen and Boehmerle, 2020).

Results

Bortezomib does not induce cognitive impairment in patients

Patients with Myasthenia gravis and systemic lupus erythematodes each received a cumulative dose of 5.2–10.4 mg m^{-2} (9.4 ± 2.1 mg m^{-2}). Patient characteristics are presented in Supplementary Table 1. We did not observe any differences in verbal learning and memory or semantic fluidity (Wilcoxon matched-pairs signed rank test, $P = 0.53$ and $P = 0.69$, respectively; Fig. 1A and B) after BTZ treatment. Visuo-spatial learning and memory were similar between baseline and follow-up assessments (Wilcoxon matched-pairs signed rank test, $P = 0.19$; Fig. 1C). There was an improvement in executive function in the Stroop test in the follow-up visit compared with baseline examination [Wilcoxon matched-pairs signed rank test, pre: 133.2 (95% confidence interval

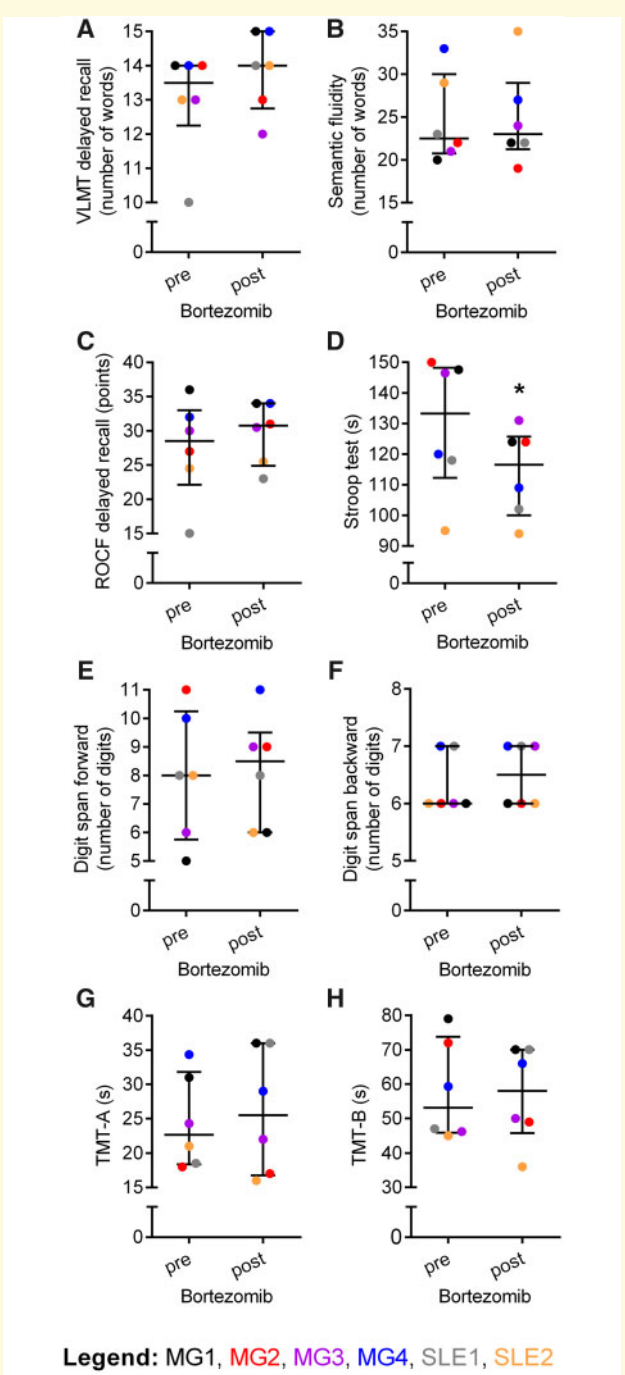


Figure 1 The influence of BTZ on cognition in patients with myasthenia gravis and systemic lupus erythematodes. (**A**) Verbal memory assessed with the verbal learning and memory test, (**B**) semantic fluidity and (**C**) visuo-spatial memory examined with the Rey–Osterrieth complex figure test were unaffected by BTZ treatment. (**D**) Executive function investigated with the Stroop test improved slightly at the follow-up time point after BTZ treatment. (**E** and **F**) Working memory was assessed with the digit span test and did not differ between baseline and follow-up examination. (**G** and **H**) Concentration, alertness and processing speed as well as multiple tasking capabilities were measured with the trail making test and were not affected by BTZ treatment. Statistical analysis: (**A–H**) paired Wilcoxon test of $n = 6$ patients for each time point. *$P < 0.05$.

(CI) 106.3–152.7), post: 116.5 (95% CI 98.7–129.3), $P = 0.0313$; Fig. 1D], which we attributed to practice effects. Working and short-term memory remained unchanged by BTZ (Wilcoxon matched-pairs signed rank test, $P > 0.99$; Fig. 1E and F). Concentration, processing speed and multi-tasking capabilities did not change after BTZ treatment (Wilcoxon matched-pairs signed rank test, $P = 0.91$ and $P = 0.69$, respectively; Fig. 1G and H). Changes in cognitive performance after BTZ treatment compared with baseline did not correlate with age or gender (data not shown). Rasch-based depression screening indicated only one patient with possible depressive symptoms consistently throughout the BTZ treatment, but the average of patients scored well below the cut-off of 12 points (Wilcoxon matched-pairs signed rank test, $P > 0.99$; Supplementary Fig. 1).

CNS penetration of bortezomib is low in mice and humans and well below toxic concentrations

To investigate whether BTZ penetrates the blood–brain barrier, we measured BTZ concentrations in the human CSF at various time points. We observed maximum serum levels of 2 nM (Fig. 2A) after single subcutaneous injection of 1.3 mg m^{-2}. BTZ levels in the CSF only reached ~7% of serum concentrations (Fig. 2B). Similar results were observed in mice, where serum concentrations peaked at 15 min after intraperitoneal injection of 0.4 mg kg^{-1} BTZ (human equivalent dose of 1.3 mg m^{-2}) but were ~15-fold higher compared with the patient (Fig. 2C). A steady state of serum concentrations was reached after 2 h in both human and mice. BTZ concentrations did not differ between the hippocampus and neocortex in murine brain tissue, but similarly to the patient only reached ~5% of serum concentrations and peaked at 1.5 nM (Fig. 2D). To determine BTZ's toxicity, we incubated adult NSC with BTZ for 2, 12, 24, 48 and 72 h. Short-term exposure of NSC (2 h) at a concentration of 68 nM reduced cell viability by 50% (non-linear regression; Fig. 2E). Longer exposure times to BTZ (≥12 h) led to a decreased IC_{50} of 3.6–9.8 nM depending on the exposure length. BTZ's toxicity did not differ between NSC and adult mature hippocampal neurons (data not shown). Short-term exposure of NSC with 1 nM BTZ did not affect caspase-3/7 activity, but higher concentrations of 50 and 100 nM produced increased caspase-3/7 activity by ~3-fold compared with vehicle treatment [Kruskal–Wallis test, 50 nM: 295.3 (95% CI 282.8–354.6), 100 nM: 332.0 (95% CI 321.0–363.5), $P < 0.0001$; Fig. 2F].

Bortezomib treatment in mice does not affect learning and memory

Adult mice treated with a human equivalent cumulative dose of 15.6 mg m^{-2} were assessed for cognitive function. BTZ-treated mice did not show any difference in visuospatial learning during the 7-day training period in the Morris water maze task where animals need to follow visual cues to swim to a hidden platform (two-way ANOVA, $P > 0.71$; Fig. 3A). In addition, we did not observe any significant differences of performance between vehicle- and BTZ-treated mice during the probe trial: neither in the latency to find the platform (Mann–Whitney *U*-test, $P = 0.90$; Fig. 3B), the time spent in each quadrant (one-way ANOVA, $P > 0.14$; Fig. 3C and D and Supplementary Fig. 2) nor the time spent in the target quadrant (*t*-test, $P = 0.12$; Fig. 3E). Recognition memory was also unaffected by BTZ as BTZ- and vehicle-treated mice spent an equal amount of time exploring a novel object (one-way ANOVA, $P = 0.99$; Fig. 3F) and spent a similar amount of time exploring (Mann–Whitney *U*-test, $P = 0.39$; Fig. 3G). In terms of working and short-term memory, the number of alternations in exploring the different arms of a Y-maze was comparable between BTZ and vehicle treatments (*t*-test, $P = 0.32$; Fig. 3H), as were the total arm entries (*t*-test, $P = 0.22$; Fig. 3I).

Discussion

Our study extends previous safety data on BTZ by three major findings. First, no signs of cognitive decline were observed in patients treated with BTZ. These data are strengthened by our findings in mice, which did not point to cognitive dysfunction even at higher cumulative dosages than used in the patients. Second, we found that BTZ shows poor blood–brain barrier penetration. We extend pre-existing data by demonstrating that CNS penetration of BTZ across the healthy blood–brain barrier is comparable between humans and mice, but much lower concentrations were observed after subcutaneous versus intraperitoneal BTZ injection as would be expected. Lastly, data from cultured primary neuronal cells indicate that doses needed to induce relevant cell damage upon short- and long-term exposure are ~5- to 10-fold higher than measured CNS concentrations. This observation is further strengthened by the fact that we could not detect an increase in caspase-3/7 activity with BTZ concentrations comparable to the C_{max} measured in the CNS. The concentration and toxicity of BTZ arguably depends highly on blood–brain barrier function. While we demonstrate that BTZ only poorly passes the *healthy* blood–brain barrier, which in part can be attributed to active transporter mechanisms such as p-glycoproteins (Foran *et al.*, 2016), others have reported much higher BTZ concentrations in various states of *impaired* blood–brain barrier function such as CNS tumours (Wang *et al.*, 2019) or middle cerebral artery occlusion (Yu *et al.*, 2006). As several important neuronal processes such as synaptic transmission and calcium signalling are tightly linked to proteasome function (Ramachandran and Margolis, 2017), an increased proteasome inhibition by BTZ could

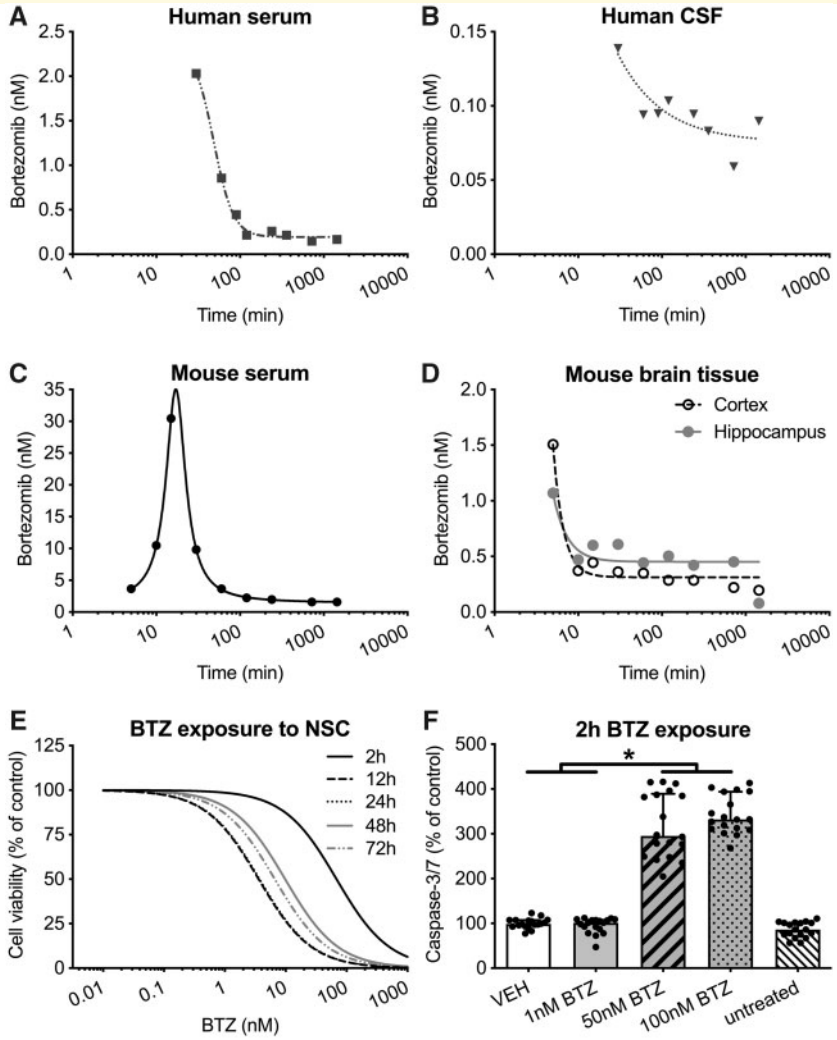


Figure 2 Pharmacokinetic profile of BTZ in human and murine serum and CNS and cell viability analysis. (**A**) BTZ serum concentration in a patient suffering from Caspr2 receptor antibody encephalitis peaked at 2 nM and reached a steady state concentration after ~2 h. (**B**) BTZ levels in the CSF did not exceed 7% of serum concentrations. (**C**) Serum BTZ concentrations in mice peaked at 15 min and again reached a steady state concentration at 2 h. (**D**) BTZ brain tissue concentrations in mice did not significantly differ between the hippocampus and neocortex and reached ~5% of serum concentrations. (**E**) BTZ concentrations ranging between 3.7 and 68 nM decreased cell viability of adult NSC by 50%. (**F**) Short-term exposure for 2 h with BTZ concentrations of 50 and 100 nM increased caspase-3/7 activity significantly, whereas smaller concentrations of 1 nM did not change caspase-3/7 activity. Statistical analysis: (**A** and **B**) $n = 2$ serum/CSF sample pairs per time point, (**C** and **D**) $n = 2$ mice per time point, (**E**) non-linear regression analysis of $n = 3$ biological replicates and (**F**) Kruskal–Wallis test with Dunn's method of $n = 3$ biological replicates. *$P < 0.05$.

have detrimental effects to the brain. Therefore, BTZ treatment should be carefully evaluated and if necessary closely monitored in patients with impaired blood–brain barrier function.

As consistent as the data seem to be, our study is not without its limitations. As the primary endpoint of the TAVAB trial was to show a decline in antibody titres after BTZ, the study did only include a limited amount of patients, no control group to compare cognitive results against and the time span between baseline and follow-up testing was rather short (2 months). While we did not see any cognitive *impairment* after BTZ, we did not observe a significant improvement in cognitive performance either, which we far more expected due to practice effects. However, in seven out of eight subtests, the median score value ranked higher at the follow-up testing suggesting that *learning* is likely not impaired by BTZ either. When tracking each individual patient's progression between the baseline and follow-up testing, there was also no clear pattern emerging of certain patients improving or worsening after BTZ treatment. Cognitive performance was not dependent on age or gender in this small cohort of patients, but clearly larger patient cohorts with longer follow-up observations such as 6 months are

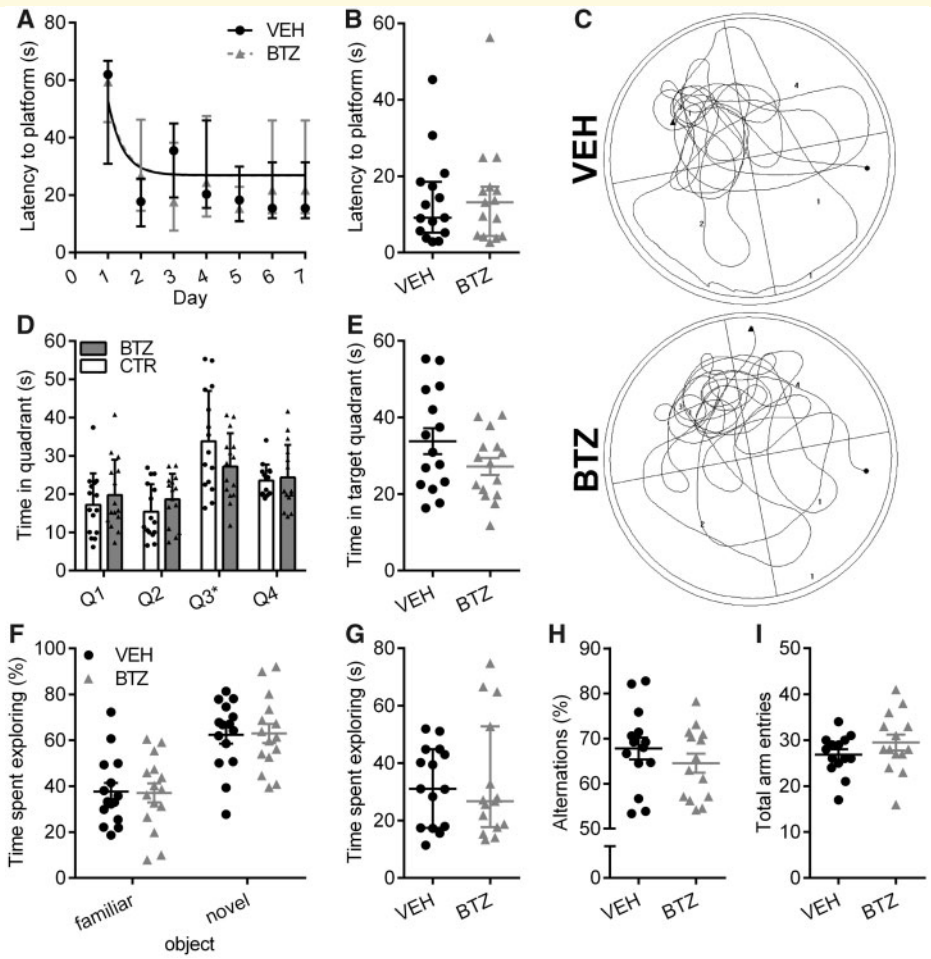


Figure 3 Effects of BTZ on cognitive function in mice. (**A**) Visuo-spatial learning was assessed with the Morris water maze task over a period of 7 consecutive days. Adult mice treated with BTZ did not show any differences in the latencies to locate the platform during the training period compared with vehicle (VEH) treatment. On Day 8 of the Morris water maze task, a single 90-s probe trial was held: (**B**) VEH- and BTZ-treated mice showed a comparable latency to reach the virtual platform. (**C**) Representative images of a VEH- and BTZ-treated mouse during the probe trial. (**D**) Both VEH- and BTZ-treated mice spent similar amounts of time in the respective quadrants (* indicates target quadrant) as well as (**E**) the target quadrant. (**F**) Mice spent comparable amounts of time exploring a novel object regardless of BTZ or VEH treatment with (**G**) overall comparable exploring behaviour. In the Y-Maze test, (**H**) neither the percentage of alternations nor (**I**) the amount of total arm entries was different between BTZ and VEH treatments. Statistical analysis: (**A**) Kruskal–Wallis test with Dunn's method and non-linear regression for $n = 15$ (VEH) and $n = 15$ (BTZ) mice, (**B** and **G**) Mann–Whitney *U*-test, (**D** and **F**) one-way ANOVA with Sidak *post hoc* and (**E**, **H** and **I**) Student's *t*-test, all $n = 15$ (VEH) and $n = 15$ (BTZ). *$P < 0.05$.

needed to corroborate these preliminary findings. Furthermore, we did not see any negative effects of prior immunosuppressive treatments such as methotrexate and cyclophosphamide that can impair cognitive function.

Our analysis of cognitive performance in the TAVAB study included two patients with systemic lupus erythematodes. Systemic lupus erythematodes *per se* seems to be associated with albeit small but relevant cognitive dysfunction, particularly for visual attention, immediate visual memory, visual reasoning and 'cognitive fluency' that seem to also be present in patients without neuropsychiatric involvement of the systemic lupus erythematodes (Leslie and Crowe, 2018). In our two patients with systemic lupus erythematodes, cognitive performance was measured at the lower end of the observed spectrum in five out of eight subtests but not reduced compared with age- and education-matched norm values (*z*-scores). However, systemic lupus erythematodes-associated cognitive dysfunction should be kept in mind when evaluating a potential BTZ treatment for systemic lupus erythematodes, as there clearly could be added negative effects.

In conclusion, our data demonstrate that CNS penetration of BTZ is low in patients and mice with an intact blood–brain barrier and that BTZ can be considered a safe medication with regard to CNS adverse effects, which is an important finding for clinicians using on and off-label BTZ therapy.

Supplementary material

Supplementary material is available at *Brain Communications* online.

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Competing interests

The authors report no competing interests.

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